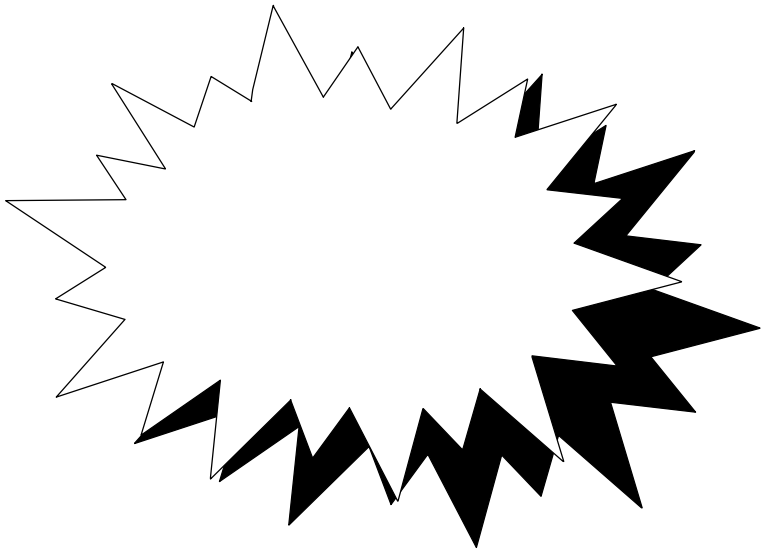

Chemical Analysis and Testing Task

Laboratory Analytical Procedure

LAP-003

Procedure Title: **Determination of Acid-Insoluble Lignin in Biomass**

Author: David Templeton and Tina Ehrman

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Determination of Acid-Insoluble Lignin in Biomass

Laboratory Analytical Procedure #003

1. Introduction

1.1 Biomass is composed largely of cellulose, a polymer of glucose; hemicellulose, a complex polymer of which the main chain consists primarily of xylans or glucomannans; and lignin, a complex phenolic polymer. Unlike the other cell wall components of biomass, the lignin is mostly insoluble in mineral acids. For this reason, lignin can be analyzed gravimetrically after hydrolyzing the cellulose and hemicellulose fractions with sulfuric acid.

1.2 This method contains two different procedures for determining acid-insoluble lignin. Both approaches have been shown to give equivalent results. Procedure A presents an approach where the acid-insoluble lignin procedure also generates the solutions required for total carbohydrate and acid-soluble lignin determinations, thereby making possible the "summative" analysis of the same sample. Procedure B is a modification of the classic "Klason lignin" determination. Although the filtrate generated from this procedure can be used to determine acid-soluble lignin, total carbohydrates should be determined on a completely separate sample.

1.3 This procedure has been adopted by ASTM as an ASTM Standard Test Method.

2. Scope

2.1 This test method covers the determination of acid-insoluble lignin of hard and soft woods, herbaceous materials (such as switchgrass and sericea), agricultural residues (such as corn stover, wheat straw, and bagasse), wastepaper (such as office waste, boxboard, and newsprint), acid and alkaline pretreated biomass, and the solid fraction of fermentation residues. All results are reported relative to the 105^{o}C oven-dried weight of the sample. In the case of extracted materials, the results may also be reported on an extractives-free basis.

2.2 The residue collected contains the acid-insoluble lignin and any condensed proteins from the original sample. An independent nitrogen analysis would be required to determine the acid-insoluble lignin content

separate from the condensed protein fraction and is outside the scope of this procedure.

2.3 All analyses shall be performed according to the guidelines established in the Ethanol Project Quality Assurance Plan (QAP).

3. References

3.1 Ehrman, C.I., and M.E. Himmel. 1994. "Simultaneous Saccharification and Fermentation of Pretreated Biomass: Improving Mass Balance Closure." *Biotechnology Techniques*, 8(2):99-104.

3.2 Moore, W.E., and D.B. Johnson. 1967. *Procedures for the Chemical Analysis of Wood and Wood Products.* Madison, WI: U.S. Forest Products Laboratory, U.S. Department of Agriculture.

3.3 NREL CAT Task Laboratory Analytical Procedure #001, "Standard Method for the Determination of Total Solids in Biomass".

3.4 NREL CAT Task Laboratory Analytical Procedure #002, "Two Stage Sulfuric Acid Hydrolysis for Determination of Carbohydrates".

3.5 NREL CAT Task Laboratory Analytical Procedure #004, "Determination of Acid-Soluble Lignin in Biomass".

3.6 NREL CAT Task Laboratory Analytical Procedure #010, "Standard Method for the Determination of Extractives in Biomass".

3.7 TAPPI Test Method T222 om-88, "Acid-Insoluble Lignin in Wood and Pulp." *In Tappi Test Methods.* Atlanta, GA: Technical Association of the Pulp and Paper Industry.

3.8 TAPPI Test Method T264 om-88, "Preparation of Wood For Chemical Analysis." *In Tappi Test Methods.* Atlanta, GA: Technical Association of the Pulp and Paper Industry.

3.9 Vinzant, T.B., L. Ponfick, N.J. Nagle, C.I. Ehrman, J.B. Reynolds, and M.E. Himmel. 1994. "SSF Comparison of Selected Woods From Southern Sawmills." Appl. Biochem. Biotechnol., 45/46:611-626.

4. Terminology

3.10 Acid-insoluble lignin is defined to be the residue, corrected for acid-insoluble ash, retained on a medium porosity filter crucible after the primary 72% and secondary 4% H_2SO_4 hydrolysis steps described in this procedure.

5. Significance and Use

3.11 The acid-insoluble lignin content is used in conjunction with other assays to determine the total composition of biomass samples.

6. Interferences

3.12 The results of acid-insoluble lignin analysis are affected by incomplete hydrolysis of biomass. Unless the sample is hydrolyzed completely, the results will be biased high. Take care to mix the acid/biomass slurry thoroughly at the beginning and periodically throughout the concentrated acid hydrolysis.

3.13 The results of acid-insoluble lignin analysis are affected by the timing of the acid digestion steps. The insoluble lignin will slowly dissolve into solution in an irreproducible fashion. The timing within this procedure must be followed closely.

3.14 Some proteinaceous materials can also form acid-insoluble substances that are collected with acid-insoluble lignin.

7. Apparatus

3.15 Analytical balance readable to 0.1 mg.

3.16 Convection oven with temperature control of $105 \pm 3^{o}C$.

3.17 Muffle furnace: an electric furnace is recommended for igniting the sample. The furnace should be fitted with an indicating pyrometer or thermocouple, so that the required temperature of $575 \pm 25^{o}C$ can be maintained.

3.18 Autoclave capable of maintaining $121 \pm 3^{o}C$ (Procedure A) or heating manifold equipped with reflux condensers with 24/40 ground glass joints (Procedure B).

3.19 Water bath set at $30 \pm 1^{o}C$ (Procedure A).

3.20 Filtration set-up including vacuum source and vacuum adapters for crucibles.

3.21 Desiccator containing anhydrous calcium sulfate.

8. Reagents and Materials

3.22 Reagents

3.22.1 72% w/w H_2SO_4 (12.00 ± 0.02 M or specific gravity 1.6389 at $15.6^{o}C/15.6^{o}C$).

3.22.2 Water, 18 megohm deionized.

3.23 Materials

3.23.1 Glass test tubes, 16x100 mm (Procedure A) or 20x150 mm (ProcedureB).

3.23.2 125 mL glass serum bottles, crimp top style, with rubber stoppers and aluminum seals to fit (Procedure A).

3.23.3 Erlenmeyer flask, 1000 mL, with 24/40 ground glass joint (Procedure B).

3.23.4 Filtration flask, 250 mL (Procedure A) or 1000 mL (Procedure B).

3.23.5 30 mL (Procedure A) or 50 mL (Procedure B) glass filtering crucible, medium porosity, nominal maximum pore size of 10 μm.

9. ES&H Considerations and Hazards

3.24 Follow all applicable NREL Laboratory Specific Hygiene Plan guidelines.

3.25 72% H_2SO_4 is very corrosive and must be handled carefully.

3.26 Use caution when handling glass bottles after the autoclave step in Procedure A, as they may have become pressurized.

10. Sampling, Test Specimens, and Test Units

3.27 Test specimens suitable for analysis by this procedure are as follows:

- biomass feedstocks, dried and reduced in particle size if necessary,
- pretreated biomass, washed free of any residual acid or alkali,
- the solids fraction of fermentation residues.

3.28 The sample must not contain particles larger than 1 mm in diameter. If milling is required to reduce the particle size of the test specimen, a laboratory mill equipped with a 40 mesh (or smaller) screen should be used.

3.29 The total solids content of the "as received" test specimen (prior to any drying or extraction steps) must be determined by Laboratory Analytical Procedure #001, "Determination of Total Solids in Biomass", in parallel with the lignin analysis. Record this value as $T_{as\ received}$.

3.30 Material with a total solids content less than 85%, on a 105°C dry weight basis, will require drying by lyophilization, oven drying, or air drying prior to milling or analysis. The amount of moisture lost as a result of the preparation procedure must be determined. This moisture content is used to calculate the total solids content of the sample based on its preparation and is recorded as T_{prep}. This value is used to correct the weight of the prepped material used in the lignin analysis, as described in the calculations section. The prepared sample should be stored in a manner to ensure its moisture content does not change prior to analysis.

> Note: Preparing samples for analysis by oven drying can produce hard chunks of material. This material must then be milled to reduce the size of the large pieces to less then 1 mm in diameter. The sample is then redried prior to testing.

3.31 Some samples may require extraction prior to analysis, to remove components that may interfere with the analysis. Laboratory Analytical Procedure #010, "Standard Method for the Determination of Extractives in Biomass", is used to prepare extractives-free sample with a moisture content suitable for lignin analysis. As part of this procedure, the percent extractives in the prepared sample, on a 105°C dry weight basis, is determined. This value, recorded as % extractives, is used to convert the % lignin reported on a extractives-free basis to an as received (whole sample) basis.

3.32 The test specimen shall consist of approximately 0.3 g of sample for Procedure A or approximately 1.0 g of sample for Procedure B. The test specimen shall be obtained in such a manner to ensure that it is representative of the entire lot of material being tested.

11. Procedure A - Summative Analysis

3.33 This procedure is suitable for air-dried, lyophilized, and extracted biomass samples, as well as for samples that have been oven dried at a temperature of 45°C or less. It is not suitable for samples that have been dried at a temperature exceeding 45°C.

> Note: The total solids content of the original sample, $T_{as\ received}$, as well as the total solids content determined as the sample is prepared, T_{prep}, must be known.

3.34 Individually label the crucibles needed for analysis, and ignite them at 575 " 25°C to achieve a constant weight of " 0.3 mg. Store the ignited crucibles in a desiccator until needed.

Note: In order to determine the absolute amounts of acid-insoluble residue and acid-insoluble ash, for quality control purposes, it is useful to weigh and record the ignited crucible to the nearest 0.1 mg.

3.35 Weigh 0.3 ± 0.01 g prepared sample to the nearest 0.1 mg and place in a 16x100 mm test tube. Record as W_1, the initial sample weight. Each sample must be run in duplicate, at minimum.

3.36 Samples for total solids determination (LAP-001) must be weighed out at the same time as the samples for the acid-insoluble lignin determination. If this is done later, it can introduce an error in the calculation because ground biomass can rapidly gain or lose moisture when exposed to the atmosphere. Record the average total solids value as T_{final}.

3.37 Add 3.00 ± 0.01 mL (4.92 ± 0.01 g) of 72% H_2SO_4 and use a glass stirring rod to mix for 1 minute, or until the sample is thoroughly wetted.

3.38 Place the test tube in the water bath controlled to 30 ± 1°C and hydrolyze for 2 hours.

3.39 Stir the sample every 15 minutes to assure complete mixing and wetting.

3.40 Transfer the hydrolyzate to a glass bottle and dilute to a 4% acid concentration by adding 84.00 ± 0.04 mL water, or by bringing the combined weight of sample, acid, and water up to 89.22 ± 0.04 g. Be careful to transfer all the residual solids along with the hydrolysis liquor.

3.41 Stopper each of the bottles and crimp aluminum seals into place.

3.42 Set the autoclave to a liquid vent cycle to prevent loss of sample from the bottle in the event of a loose crimp seal. Autoclave the samples in their sealed bottles for 1 hour at 121 ± 3°C.

3.43 After completion of the autoclave cycle, allow the samples to cool for about 20 minutes at room temperature before removing the seals and stoppers.

3.44 Vacuum filter the hydrolysis solution through one of the previously ignited filtering crucibles.

3.45 If a carbohydrate analysis (LAP-002) and/or an acid-soluble lignin analysis (LAP-004) is desired, decant 15-25 mL of filtrate into a resealable container. If this aliquot is not used immediately for further analysis, store in refrigerator at 4°C.

Note: Acid-soluble lignin should be analyzed within 24 hours, preferably within 6 hours of hydrolysis.

3.46 Use hot deionized water to wash any particles clinging to the glass bottle into the crucible and to wash the filtered residue free of acid using vacuum filtration.

3.47 Dry the crucible and contents at $105 \pm 3^{o}C$ for 2 hours or until constant weight is achieved ("0.3 mg upon reheating).

3.48 Cool in desiccator and record the weight, W_2, the weight of the crucible, acid-insoluble lignin, and acid-insoluble ash to the nearest 0.1 mg.

3.49 Place the crucible and contents in the muffle furnace and ignite at $575 \pm 25^{o}C$ for a minimum of 3 hours, or until all the carbon is eliminated. Heat at a rate of $10^{o}C$/min to avoid flaming. If the sample tends to flare up, the container should be partially covered during this step. Avoid heating above the maximum stated temperature. Protect the test container from strong drafts at all times to avoid mechanical loss of the test specimen.

3.50 Cool in desiccator and record the weight, W_3, the weight of the crucible and acid-insoluble ash, to the nearest 0.1 mg.

Note: The amount of acid-insoluble ash remaining in the crucible is not equal to the total ash content of the sample. Refer to Laboratory Analytical Procedure #005 if total ash is to be determined.

12. Procedure B - Klason Lignin Determination

3.51 This procedure is suitable for oven-dried samples (including those dried at temperatures between $45^{o}C$ and $105^{o}C$) as well as air-dried, lyophilized, and extracted biomass samples.

Note: The total solids content of the original sample, $T_{as\ received}$, as well as the total solids content determined as the sample is prepared, T_{prep}, must be known.

3.52 Individually label the crucibles needed for analysis, and ignite them at $575 \pm 25^{o}C$ to achieve a constant weight of $\pm$ 0.3 mg. Store the ignited crucibles in a desiccator until needed.

Note: In order to determine the absolute amounts of acid-insoluble residue and acid-insoluble ash, for quality control purposes, it is useful to weigh and record the ignited crucible to the nearest 0.1 mg.

3.53 Weigh 1.0 ± 0.05 g prepared sample to the nearest 0.1 mg and place in a 20x150 mm test tube. Record as W_1, the initial sample weight. Each sample must be run in duplicate, at minimum.

3.54 Samples for total solids determination must be weighed out at the same time as the samples for the acid-insoluble lignin determination. If this is done later, it can introduce an error in the calculation because ground biomass can rapidly gain or lose moisture when exposed to the atmosphere. Record the average total solids value as T_{final}.

3.55 Add 15.00 ± 0.02 mL of 72% H_2SO_4, chilled to 4°C in the refrigerator. Use a glass stirring rod to mix for 1 minute, or until the sample is thoroughly wetted.

3.56 Hydrolyze the sample for 2 hours at room temperature (approximately 20°C), stirring every 15 minutes to assure complete mixing and wetting.

3.57 Transfer the hydrolyzate to a 1000 mL Erlenmeyer flask and dilute to a 3% acid concentration with 560 mL of deionized water. Be careful to transfer all the residual solids along with the hydrolysis liquid.

3.58 Place the flask on the heating manifold and attach to the reflux condenser. Heat the liquid to a gentle boil. Start timing at the onset of boiling, and reflux for 4 hours ± 5 minutes.

3.59 At the end of 4 hours, rinse the condenser with a small amount of deionized water before disassembling reflux apparatus.

3.60 Vacuum filter the hydrolysis solution through one of the previously ignited filtering crucibles.

3.61 If an acid-soluble lignin determination (LAP-004) is to be run, record the weight of the collected filtrate. Decant 15-25 mL of filtrate into a resealable container. If this aliquot is not used immediately for further analysis, store in refrigerator at 4°C.

> Note: Acid-soluble lignin should be analyzed within 24 hours, preferably within 6 hours of hydrolysis.

3.62 Use hot deionized water to wash any particles clinging to the glass bottle into the crucible and to wash the filtered residue free of acid using vacuum filtration.

3.63 Dry the crucible and contents at 105 ± 3°C for 2 hours or until constant weight is achieved (±0.3 mg upon reheating).

3.64 Cool in desiccator and record the weight, W_2, the weight of the crucible, acid-insoluble lignin, and acid-insoluble ash to the nearest 0.1 mg.

3.65 Place the crucible and contents in the muffle furnace and ignite at 575 ± 25^{o}C for a minimum of 3 hours, or until all the carbon is eliminated. Heat at a rate of 10^{o}C/min to avoid flaming. If the sample tends to flare up, the container should be partially covered during this step. Avoid heating above the maximum stated temperature. Protect the test container from strong drafts at all times to avoid mechanical loss of the test specimen.

3.66 Cool in desiccator and record the weight, W_3, the weight of the crucible and acid-insoluble ash, to the nearest 0.1 mg.

Note: The amount of acid-insoluble ash remaining in the crucible is not equal to the total ash content of the sample. Refer to Laboratory Analytical Procedure #005 if total ash is to be determined.

13. Calculations

3.67 For lyophilized, air dried, or oven dried samples, or samples requiring no preparation, calculate % acid-insoluble lignin on an as received 105^{o}C dry weight basis as follows:

$$\%\ acid\text{-}insoluble\ lignin = \frac{W_2 - W_3}{W_1 \times \frac{T_{asreceived}}{T_{prep}}} \times 100\% = \frac{W_2 - W_3}{W_1 \times \frac{T_{final}}{100}\%} \times 100\%$$

Where:

W_1 = initial sample weight.
W_2 = weight of crucible, acid-insoluble lignin, and acid-insoluble ash.
W_3 = weight of crucible and acid-insoluble ash.
$T_{as\ received}$ = % total solids content of the original sample (prior to any preparation steps) on a 105^{o}C dry weight basis, as determined by the LAP-001.
T_{prep} = % total solids content of the sample as determined during the preparation of the sample for analysis (by lyophilization, oven-drying, or air drying).
T_{final} = % total solids content of the prepared sample used in this lignin analysis, on a 105^{o}C dry weight basis, as determined by the LAP-001.

Note: If the sample used in the acid-insoluble lignin analysis required no preparation (analyzed as received), then T_{prep}=100% and T_{final}=$T_{as\ received}$. If a sample was prepared by drying at 105^{o}C prior to analysis, then T_{prep}=$T_{as\ received}$ and T_{final}=100%.

3.68 For an extracted sample, it may be necessary to report the results on an extractives-free

105°C dry weight basis or on an as received (whole sample) 105°C dry weight basis, or both.

3.68.1 Calculate % acid-insoluble residue on an extractives-free basis as follows:

$$\%\ acid\text{-}insoluble\ residue_{extractives\text{-}free} = \frac{W_2 - W_3}{W_1 \times \frac{\%T_{final}}{100\%}} \times 100\%$$

Where:
W_1 = initial weight of extracted sample
W_2 = weight of crucible, acid-insoluble residue, acid-insoluble ash
W_3 = weight of crucible and acid-insoluble ash
$\%T_{final}$ = % total solids of the extracted sample determined at 105°C as described by the Standard Method for the Determination of Total Solids in Biomass.

3.68.2 Correct the acid-insoluble residue value calculated above on an extractives-free basis, to an as received (whole sample) 105°C dry weight basis:

$$\%\ acid\text{-}insoluble\ residue_{whole\ sample} = \%\ AIR_{extractives\text{-}free} \times \frac{(100\% - \%\ extractives)}{100\%}$$

Where:
$\%\ AIR_{extractives\text{-}free}$ = % acid-insoluble residue on an extractives-free 105°C dry weight basis, as determined in the previous step
$\%\ extractives$ = % extractives in the sample extracted as described in the Standard Method for the Determination of Extractives in Biomass.

14. Report

3.69 Report the percent acid-insoluble lignin, to two decimal places, on a 105°C dry weight basis, and cite the reporting basis.

3.70 For replicate analyses of the same sample, report the average, standard deviation, and relative percent difference (RPD).

15. Precision and Bias

3.71 Data obtained by replicate testing of a hybrid poplar in one laboratory gave a standard deviation in Klason lignin content of 0.32% and a CV% of 1.26%.

3.72 Data obtained by replicate testing of a hybrid poplar sample in six different laboratories gave a standard deviation of 2.37% and a CV% of 9.92%.

16. Quality Control

3.73 *Reported significant figures:* The acid-insoluble lignin results will be reported with two decimal places, on a 105^{o}C dry weight basis.

3.74 *Replicates:* All samples and all method verification standards are to be analyzed in duplicate, at minimum.

3.75 *Blank:* A blank crucible is to be run through the analysis. The dish is to be weighed empty, ashed and reweighed. The difference in weight must be less than the equivalent of a 0.5% error.

3.76 *Relative percent difference criteria:* The RPD must be less than 3.4%. If the RPD is too large, the sample will be rerun.

3.77 *Method verification standard:* A method verification standard must be run in duplicate with every batch.

3.78 *Calibration verification standard:* Not applicable.

3.79 *Sample size:* Approximately 0.6 grams of sample is required for conducting duplicate analyses by Procedure A. Two grams will be required for Procedure B. If there is insufficient sample, the result will be flagged and the lack of precision will be noted.

3.80 *Sample storage:* Wet samples, prior to preparation, must be stored in the refrigerator. Samples that have been prepped by extraction, lyophilization, or oven drying must be stored in tightly sealed containers or in a desiccator.

3.81 *Standard storage:* Not applicable.

3.82 *Standard preparation:* Not applicable.

3.83 *Definition of a batch:* Any number of samples which are analyzed together and

recorded together. Samples within a batch must be of the same matrix. The maximum size of a batch would be limited by the equipment constraints. A batch cannot be larger than what is practical with the equipment.

3.84 *Control charts:* The result of each replicate analysis of the method verification standard is recorded along with the average, RPD, and a laboratory book/page reference. The average value obtained for each analysis of the method verification standard is to be control charted.